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Identification of Vaccine Resistant Isolates of *Bacillus anthracis*

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In conducting the research described in this report, the investigators adhered to the "Guide for the Care and Use of Laboratory Animals", as promulgated by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council. The facilities are fully accredited by the American Association for Accreditation of Laboratory Animal Care.

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ABSTRACT

Several strains of Bacillus anthracis have been reported previously to cause fatal infection in immunized guinea pigs. In this study, guinea pigs were immunized with either a protective antigen vaccine or a live Sterne strain spore vaccine, then challenged with virulent B. anthracis strains isolated from various host species from the United States and foreign sources. Confirmation of the previously reported studies (which used only protective antigen vaccines) was made with the identification of 9 of the 27 challenge isolates as being vaccine resistant. However, guinea pigs immunized with the live Sterne strain spore vaccine were fully protected against these 9 isolates. In experiments designed to determine the basis of vaccine resistance, guinea pigs which were immunized with individual toxin components and which demonstrated enzyme-linked immunosorbent assay antibody titers comparable to those induced by Sterne strain spore vaccine were not protected when challenged with a vaccine-resistant isolate. We concluded that antibodies to toxin components may not be sufficient to provide protection against all strains of B. anthracis, and that other antigens may play a role in active immunity. As a practical matter, it follows that the efficacy of anthrax vaccines must be tested by using vaccine-resistant isolates if protection against all possible challenge strains is to be assured.

INTRODUCTION

Two virulence factors have been described for Bacillus anthracis, each of which is associated with a separate plasmid (11, 17, 28). The capsule, composed of poly-D-glutamic acid, inhibits phagocytosis (14, 32) and is nonimmunogenic (22). Anthrax toxin, readily obtained from culture supernatants (20), is composed of edema factor (EF), protective antigen (PA), and lethal factor (LF) (2, 23, 25). The individual toxin components show no biological activity in experimental animals. A PA-LF combination produces lethality after i.v. injection in some species (2, 25) and a PA-EF mixture causes edema when injected s.c. (8, 25). The production of both capsule and toxin are required for the bacterium to be fully virulent.

An excellent review of the various anthrax vaccines and immunogen preparations has been published by Hambleton et al. (12). Live, attenuated spore vaccines are licensed currently only for veterinary use in the United States and have been shown for some time to be effective in protecting livestock (10, 13, 18, 26). At least three different chemical-type vaccines, prepared from cell-free filtrates, have been used in human trials (4, 6, 19, 31). The predominant component in these cell-free filtrates is PA. The commercial product licensed for human use in the United States is supplied by the Michigan Department of Public Health, and will be referred to as PA vaccine. This vaccine is not a highly purified product and has been reactogenic in some recipients (4).

Although various antigen preparations appear to provide a substantial degree of protection when immunized animals are challenged with the standard Vollum strain, early studies by Auerbach and Wright (1) and Ward et al. (29) demonstrated that certain B. anthracis isolates were able to override this immunity. The current study, undertaken as part of an overall effort to evaluate and improve the chemical vaccine presently used for humans, seeks to confirm and expand upon those early studies. Furthermore, a better understanding of the virulence of B. anthracis should result from the elucidation of vaccine resistance among strains of the bacillus.

MATERIALS AND METHODS

Animals. Female, Hartley guinea pigs, weighing 300 to 350 g at the beginning of the immunizations, were used for this study.

Protection Studies. The chemical anthrax vaccine is prepared commercially by the Michigan Department of Public Health by adsorbing a V770-NP1-R culture filtrate to aluminum hydroxide gel. Filtration of the culture through sintered glass filters removes most of the LF and EF toxin components. The major toxin component of the PA vaccine is, therefore, PA . The vaccine was administered i.m. in three 0.5-ml doses at 2-week intervals. The commercial live, veterinary, Sterne-strain spore vaccine (Burroughs Wellcome Company) was administered in 3 doses: 0.2, 0.3, and 0.5 ml i.m. at 2-week intervals. The stock spore vaccine contained 5-6 x 10^6 spores/ml. According to the

manufacturer's recommendations, either 1.0 ml (cattle, horses, mules) or 0.5 ml (sheep, swine, goats) as a single dose or two doses are protective. Controls for each experiment received physiological saline according to the corresponding immunization protocol.

We prepared purified toxin components, LF and EF (16), for use as immunogens by adsorbing each to aluminum hydroxide gel (Alhydrogel, Accurate Chemical and Scientific Corp., Westbury, New York), following a procedure similar to that used for the preparation of the PA vaccine (19). Briefly, toxins were adsorbed to a 1% suspension of aluminum hydroxide gel at pH 5.9 with stirring for 3 days at 4°C. Absorbance values at 280nm indicated that 93%, 87%, and 57% of PA, LF, and EF toxin components, respectively, had been adsorbed to the gel. The final product was resuspended in phosphate-buffered saline (PBS) (2.3 mM sodium phosphate, monobasic; 7.5 mM sodium phosphate, dibasic; 0.15M sodium chloride; pH 7.3) to yield 40 µg protein per ml. Guinea pigs were immunized i.m. at 2-week intervals with three 0.5-ml doses of LF- or EF-adsorbed antigens either alone or concomitantly with 0.5-ml of PA vaccine given in the opposite flank.

Two weeks after the last immunization dose, serum was collected for titers and the animals were challenged.

Challenge Isolates. Strains of B. anthracis used for challenges were isolated from various host species, animal products or handling facilities, and from various geographical areas of the United States and from foreign sources between 1925 and 1983 (Table 1). Spore suspensions used for challenges were obtained from cultures grown at 37°C for 3 days on blood agar

plates. Spores were washed from the culture with phenolized gel phosphate buffer (28 mM sodium phosphate, dibasic; 0.2% gelatin; 1% phenol; pH 7.2). Spore suspensions were heat shocked at 60°C for 30 min, washed, resuspended in phenolized gel phosphate, and held at 4°C until diluted for challenge. A standard challenge dose of 1000 spores in 0.5 ml administered i.m. 14 days after the last immunization dose was used throughout unless noted.

Enzyme-linked immunosorbent assay (ELISA). Purified PA, LF, or EF toxin components (16) were diluted to 1µg/ml with 0.05 M sodium borate buffer (pH 9.5), 100µl added to each well of 96-well microtiter plates (Linbro), and incubated overnight at 4°C. Plates were incubated for 30 min at room temperature (RT) with 200 µl PBS+0.5% gelatin (PBSG), washed two times with PBS, and frozen at -70°C in freezer bags. Before use, plates were washed three times with PBS+0.05% Tween 20 (PBST) and 100 µl sample volumes were added per well. Dilutions were made with PBSG. After incubating overnight at either 4°C or 2 h at 37°C, plates were washed three times with PBST and incubated for 2 h at RT with horse-radish peroxidase conjugated to Staphylococcal Protein A (HRP-Protein A; Sigma) at a 1/5000 dilution. For color development, plates were washed five times with PBST and incubated for 20 min at RT with ABTS (2,2'-Azino-bis(3-ethylbenzthiazolinesulfonic acid, Sigma) at 1 mg per ml in 0.1 M sodium citrate buffer, pH 4.0 +0.003% hydrogen peroxide. The reaction was stopped by adding 100 µl of 10% sodium dodecyl sulfate (SDS) to each well. Plates were read on a Dynatech Microelisa Auto Reader MR580 (Dynatech Instruments, Inc.) at a wavelength of 405 nm. Positives were scored as those wells giving a reading of >0.300.

RESULTS AND DISCUSSION

Protection of animals immunized against B. anthracis is usually demonstrated by challenging with the Vollum strain, the proposed neotype culture of B. anthracis (24), or with one of its derivatives, i.e. Vollum 1B, M36, or V1b-189. It is apparent from the literature that protection against challenge with the Vollum strain or one of its derivatives can be achieved by using any one of several different cell-free preparations (3, 12, 15, 19, 30). However, results presented in Table 2, Experiment I, lists, with Vollum and Vollum 1B, those strains of B. anthracis that killed 50% or more of the PA-immunized animals. The data confirm the findings of Auerbach and Wright (1) and Ward et al. (29) that, although guinea pigs were immunized effectively against a Vollum challenge, they were not protected against challenge with some isolates of B. anthracis. Although we found 9 out of 27 isolates tested to be resistant to immunization with the PA vaccine, we cannot say that this reflects the percentage or proportion of vaccine-resistant isolates found in nature. A larger number of isolates will have to be assayed before a percentage or proportion of vaccine-resistant isolates can be determined.

Vaccination of guinea pigs with Sterne strain spores appears to provide broad protection against i.m. challenge with various anthrax isolates (Table 2, Experiment II). Three graded, immunizing doses of spores were administered to immunize the guinea pig and, at the same time, to preclude the occasional death in guinea pigs resulting from a dose of $>10^6$ Sterne spores. A dose-response curve of the Sterne spore vaccine was obtained by injecting guinea

pigs with 0.5 ml i.m. in a single dose or as 2 doses 14 days apart. The animals were then challenged i.m. with 2500 spores of Vollum 1B 2 weeks after immunization (Table 3). The data indicate that excellent protection and antibody response to PA antigen can be achieved with two immunization doses of 10^6 Sterne spores. The dose of spores administered, the strain of avirulent spores used, and the presence of adjuvants (5, 9, 10, 18, 27) are all important factors influencing the ability of a spore vaccine to protect against challenge. The greater protection afforded by a spore vaccine may be due to immunological processing of antigens available in the spore and vegetative cells after germination, or to the presence of a yet unidentified immunogen(s) not present in the chemical-type vaccine.

Antibody responses of guinea pigs immunized with PA vaccine or Sterne spore vaccine are shown in Table 4. Antibody titers demonstrate that PA was present and immunogenic in the PA vaccine. The titers against PA, LF, and EF antigens of sera obtained from guinea pigs immunized with Sterne spore vaccine suggest that protection with the PA vaccine might be enhanced by addition of LF or EF toxin components to yield a similar antibody response. When such an experiment was performed (Table 5), protection against Vollum 1B challenge was observed whenever the PA toxin component was part of the vaccine. However, no component vaccine was capable of eliciting protection against a vaccine-resistant isolate (NH), even when antibody titers approach those of Sterne spore vaccinated animals. Neither LF nor EF alone provide protection against either challenge strain. Evaluation of protection afforded by immunization against anthrax has been made by either survival tests or measurement of the

serological titer to the antigen used as an immunogen. Our results indicate that, although a high ELISA titer was obtained after immunization, as demonstrated by immunization with Sterne strain spores or PA vaccine + LF, it did not reflect the level of expected protection. This was demonstrated after challenge with a vaccine-resistant isolate. Ward et al. (29), who used an antigen preparation very similar to ours, also recorded deaths of guinea pigs with high antibody titers which were challenged with their vaccine-resistant isolate.

The apparently greater virulence among the vaccine-resistant isolates might be reflected in their LD_{50} values. Results from only two of the vaccine-resistant isolates, NH and Ames, show (Table 6) that they have LD_{50} values 8-fold less than Vollum 1B. However, these two isolates have approximately equal LD_{50} values as the Vollum isolate. Although there was an apparent decrease in virulence of our Vollum 1B strain compared to previously reported LD_{50} values for this strain (s.c. LD_{50} of <10 spores; 21) it did kill our control guinea pigs. The Vollum 1B strain also has been studied recently for quantitation of lethal toxin activity (7, 20) and plasmid isolations (11, 17). Comparison of the vaccine-resistant isolates with the Vollum cultures suggest that it is not the difference in the LD_{50} values of the isolates that determine vaccine resistance but some other factor(s) (1, 29).

This study, which compares protection of guinea pigs after immunization with either the chemical-type vaccine or spore vaccine against challenge by various B. anthracis strains, indicates the superior protective efficacy of the latter. Immunization with cell-free preparations, which contain components of the anthrax toxin, did not provide an adequate protective response against some challenge isolates of B. anthracis. The fact that the spore vaccine provided protection against all isolates tested suggests that other antigens may play a role in active immunity. Since this vaccine is a live immunogen, safety factors must be considered before its use. In evaluations of anthrax vaccines, it is important to test protection by using a wide variety of challenge isolates.

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TABLE 1. Histories of isolates used in this study

ISOLATE	SOURCE & DATE OF ISOLATION	REFERENCE
Vollum	Cow; Ca. 1944	USAMRIID[a]
Vollum 1B	Derived from Vollum	USAMRIID
Ames	Cow; Iowa 1980	USDA[b]
Buffalo	Buffalo; Iowa 1979	R. A. Packer
17T5	Kudu; South Africa 1957	VRI[c]
NH	Human; New Hampshire 1957	USAMRIID
SK31	Wildebeest; South Africa 1974	CDC[d]
ACB	Human; Ohio 1952	USAMRIID
SK61	Human; California 1976	CDC
SK162	Human; Florida 1976	CDC
VH	Human; South Africa 1952	VRI
V770	Cow; Florida 1951	USAMRIID
G28	South Africa 1939	VRI
107	Human; Haiti 1943	USAMRIID
205	Goat; South Africa 1942	USAMRIID
Nebr	Cow; Nebraska 1978	R. A. Packer
57	Goat; South Africa 1946	VRI
Albia	Iowa 1963	R. A. Packer
1928	Cow; Iowa 1925	R. A. Packer
3515	1963	R. A. Packer
SK102	Pakistan wool; New Jersey 1976	CDC
SK128	Ireland wool; Massachusetts 1976	CDC
SK465	Buffalo; Iowa 1979	CDC
C4880C	Mill; North Carolina 1978	CDC
M36	Derived from Vollum	USAMRIID
Vollum 1	South Africa 1948	VRI
TX368203	Cow, Texas 1983	TVMDL[e]

[a]USAMRIID - U.S. Army Medical Research Institute of Infectious Diseases, Ft. Detrick, Frederick, MD.

[b]USDA - U.S. Department of Agriculture, Ames, IA.

[c]VRI - Veterinary Research Institute, Republic of South Africa.

[d]CDC - Centers for Disease Control, Atlanta, GA.

[e]TVMDL - Texas Veterinary Medical Diagnostic Laboratory, College Station, TX.

TABLE 2. Vaccine efficacy against various *Bacillus anthracis* isolates

CHALLENGE ISOLATE[a]	EXPERIMENT I		EXPERIMENT II	
	SALINE	PA VACCINE	SALINE	STERNE SPORE VACCINE
VOLLUM	0/6[b]	6/6	N.D.[c]	N.D.
VOLLUM 1B	1/8	10/10	2/5	8/8
AMES	1/6	0/6	0/6	6/6
BUFFALO	1/6	1/6	0/6	5/6
17T5	0/6	1/6	0/6	7/8
NH	0/6	3/9	0/6	8/8
SK31	0/6	3/6	0/6	6/6
ACB	0/6	1/6	0/6	6/6
SK61	0/6	2/6	0/6	6/6
SK162	1/6	2/6	0/6	6/6
VH	0/6	3/6	1/6	6/6

[a]Challenge dose 10^3 spores i.m.

[b]Survivors/total.

[c]N.D. = not done.

TABLE 3. Immune response of guines pigs immunized with sterne strain spores

No. Spores Injected Per Dose[a]		% Survival	ELISA Titer[b]
Single Dose	10^4	50	14
	10^5	50	29
	10^6	70	260
Two Doses	10^4	44	36
	10^5	80	230
	10^6	90	5600
Controls	None	50	10

[a]Guinea pigs were immunized i.m. with 0.5 ml of suspensions of 10^4, 10^5 or 10^6 spores as a single dose or two doses 14 days apart. Sera were obtained and animals were challenged i.m. with 2500 spores of Vollum 1B 14 days after immunization.

[b]Average reciprocal ELISA titer to PA antigen.

TABLE 4. Antibody response to immunization

Immunization	# Animals	PA	LF	EF
PA Vaccine	74	10,000[a] (100)[b]	10 (49)	5 (26)
Sterne Spore Vaccine	35	3,000 (130)	1,000 (100)	100 (97)

[a]Reciprocal ELISA titer.

[b]Percent animals with titers.

TABLE 5. Protective efficacy of PA, LF, EF combinations

	ANTIBODY RESPONSE			CHALLENGE	
VACCINE	PA	LF	EF	VOLLUM 1B	NH
Sterne	7,800[a]	480	10	7/7[b]	12/12
PA	14,200	25	4	5/6	4/12
LF	10	2,500	4	1/6	1/6
EF	250	10	2,500	0/6	0/6
PA + LF	12,600	1,000	10	6/6	2/6
PA + EF	10,000	18	750	6/6	1/6
None	0	0	0	1/6	0/6

[a]Reciprocal ELISA Titer.

[b]Survivors/Total.

TABLE 6. LD_{50} values for three *B. anthracis* isolates

ISOLATE	LD_{50} CFU
VOLLUM	33
VOLLUM 1B	395
NH	51
AMES	49